Amplification Cut-Off Validation

Plexor® HY
AmpFlSTR® Identifiler® Plus

Report

Appendix A (Excel Spreadsheets)

Appendix B (Sensitivity Study Setup Worksheets, Results, Electropherograms)

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This study has been technically reviewed and approved for use by:

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01/22/16

Date

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Date

Internal Validation of Amplification Cut-off

Introduction

In order to keep up with the increasing demand for DNA testing in the District of Columbia, the Department of Forensic Sciences Forensic Biology Unit is investigating the use of an amplification cut-off as a part of the DNA analysis procedure. This method will allow the laboratory to confidently reduce the time and cost of processing samples which are not likely to produce useable results. The following validation will determine an appropriate overall value to stop sample processing after the quantitation step and an appropriate ratio to stop sample processing of qualifying sexual assault case samples. It includes a casework evaluation of previously quantified and amplified samples, a sensitivity study and a mixture study. The following SWGDAM requirements will be addressed: mock case samples, sensitivity, accuracy, reproducibility, contamination and mixture. Studies on precision were performed as a part of the Plexor® and Identifiler® Plus Validation.

I. Casework Evaluation

a. Objective

Use previously amplified samples to determine an appropriate amplification cutoff using Quantifiler® Duo and Identifiler®/Identifiler® Plus.

b. Materials and Methods

Previously worked cases which included samples quantified at a value of 0.04ng/µl or less were pulled for evaluation. 469 samples from 98 cases quantified with Quantifiler® Duo and amplified with Identifiler® or Identifiler® Plus for 28 cycles were examined and evaluated. 47 samples from 16 cases quantified with Quantifiler® Duo and amplified with Identifiler® Plus for 29 cycles were examined and evaluated. Data was also collected from two sets of low level mock casework samples which were quantified with Quantifiler® Duo and amplified with Identifiler® Plus as a part of two previous internship studies. In total, data was gathered from 583 samples for this study.

c. Data Analysis

An excel spreadsheet was generated to record the following information: sample, quantification value, Ct, amplification target, number of cycles, and IPC value. Electropherograms from each sample were then evaluated as mixture or single source (if able to be determined) and the number of loci, excluding amelogenin, which produced any result was recorded. Peak heights, peak height ratios and loci with a single peak below stochastic threshold were also recorded. Samples with results at 10-15 loci were colored green, 5-9 loci were colored yellow and 0-4 loci were colored red. The spreadsheet was then sorted by amplification target to determine if there was an observable trend. (See Appendix A for spreadsheet.)

d. Results

28 Cycle Amplifications

Amplified Quantity	% 10-15 loci	% 5-9 loci	% 0-4 loci
>400pg	100	0	0
350-400pg	41.66666667	25	33.33333333
300-350pg	51.61290323	9.677419355	38.70967742
250-300pg	36	16	48
200-250pg	45.45454545	24.24242424	30.3030303
150-200pg	28.81355932	25.42372881	45.76271186
100-150pg	24.05063291	18.98734177	56.96202532
50-100pg	12.82051282	14.52991453	72.64957265
0-50pg	2.873563218	7.471264368	89.65517241

Figure 1.

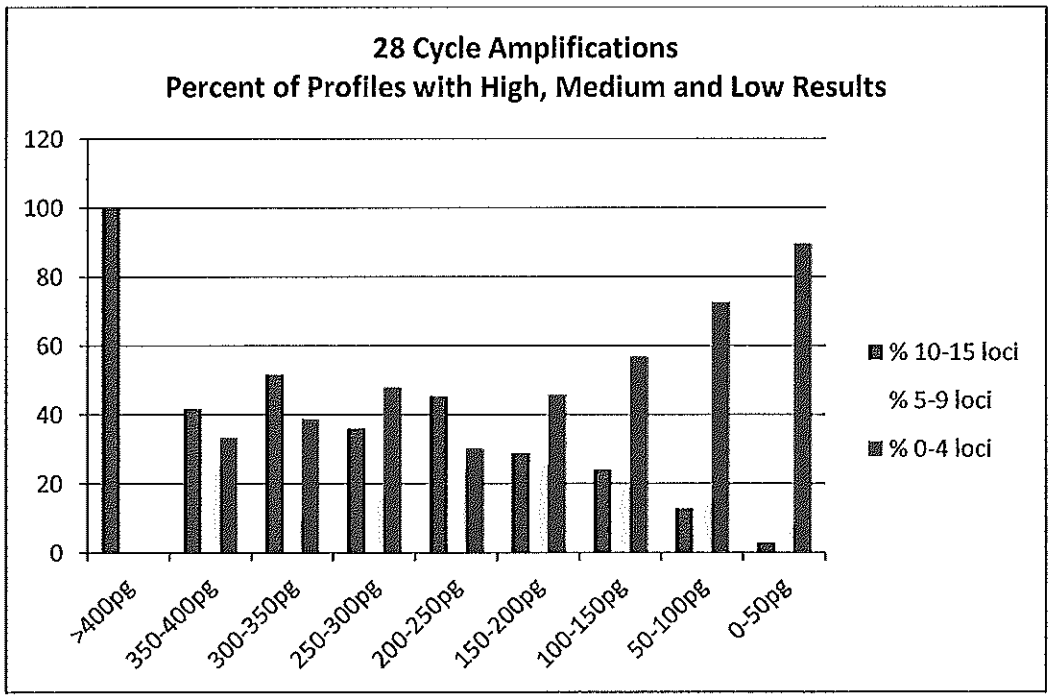

Figure 2. For the purposes of this study, samples with results at 10-15 loci will be considered good profiles, samples with results at 5-9 loci will be considered useable profiles and samples with results at 0-4 loci will be considered poor profiles. Good profiles were obtained for the majority of samples amplified at 200pg or more. More than 50% of the samples amplified at less than 200pg produced poor profiles, however good profiles were still obtained for some samples.

Quantity Amplified	Average PH (rfu)	Standard deviation
>400pg	641.67	380.91
350-400pg	215.80	54.95
300-350pg	222	77.23988607
250-300pg	157.6	66.87525701
200-250pg	194.9230769	93.28045663
150-200pg	175.5384615	107.3558067
100-150pg	139.9166667	50.66764396
50-100pg	127.7368421	49.59372956
0-50pg	123.2	53.93673543

Figure 3.

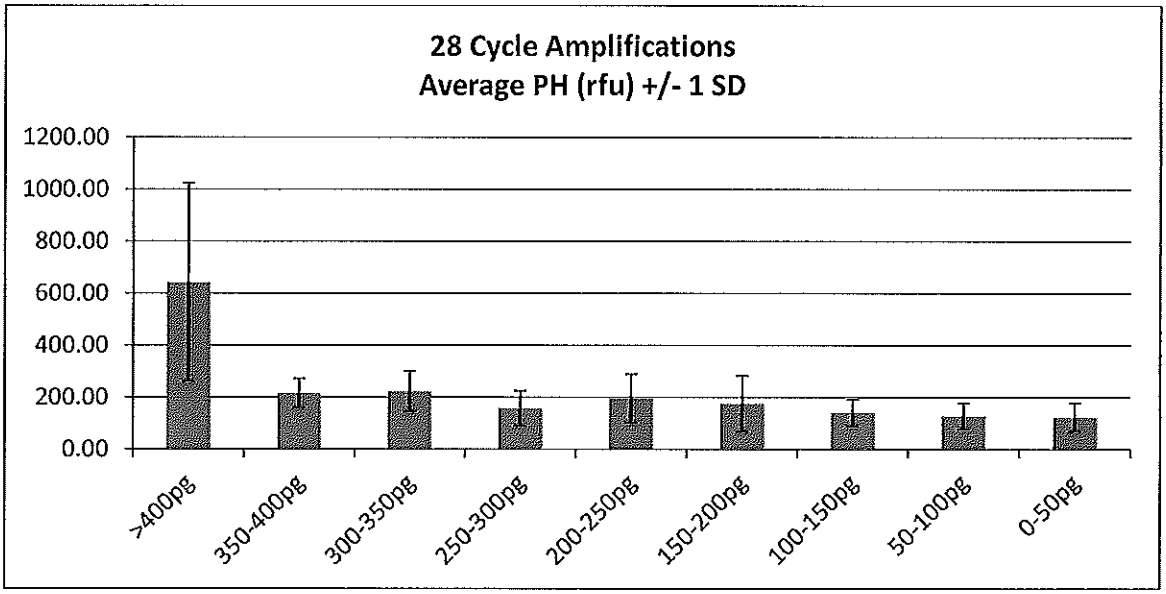

Figure 4. Average peak heights fall below stochastic threshold at 300pg, however all data was not able to be considered for calculations due to a high number of mixture profiles.

Quantity Amplified	Average Loci	standard deviation	Average-2SD	Average+2SD	Range (+/- 2SD)
>400pg	15.00	0.00	15	15	always full profile
350-400pg	8.86	6.29	-3.71528468	21.42957039	no results to full profile
300-350pg	7.69	5.65	-3.61272698	18.99203733	no results to full profile
250-300pg	6.52	6.15	-5.78338707	18.82338707	no results to full profile
200-250pg	8.67	5.54	-2.40883182	19.74216515	no results to full profile
150-200pg	6.07	5.13	-4.19001392	16.32560714	no results to full profile
100-150pg	5.30	5.28	-5.24695621	15.85455114	no results to full profile
50-100pg	3.13	4.24	-5.35520042	11.61161068	no results to 12 loci
0-50pg	1.21	2.94	-4.65907198	7.084359332	no results to 7 loci

Figure 5. Using +/- two standard deviations, good profiles may be obtained at target amplifications as low as 50pg. Below 50pg, profiles may be useable or poor.

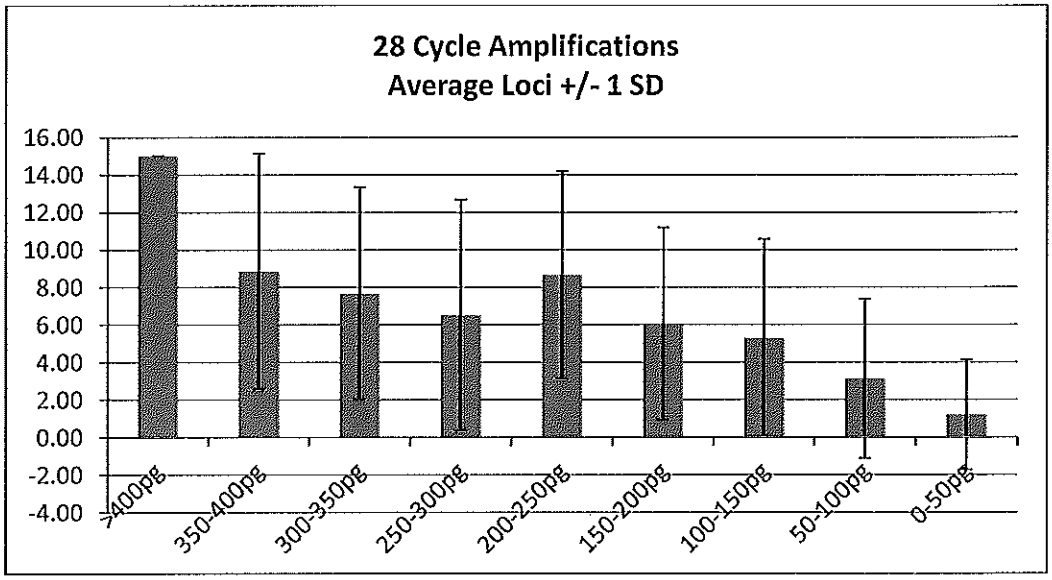

Figure 6.

29 Cycle Amplifications

Amplified Quantity	% 10-15 loci	% 5-9 loci	% 0-4 loci
300-400pg	100	0	0
200-300pg	80	0	20
100-200pg	80	6.666666667	13.33333333
0-100pg	32	32	36

Figure 7.

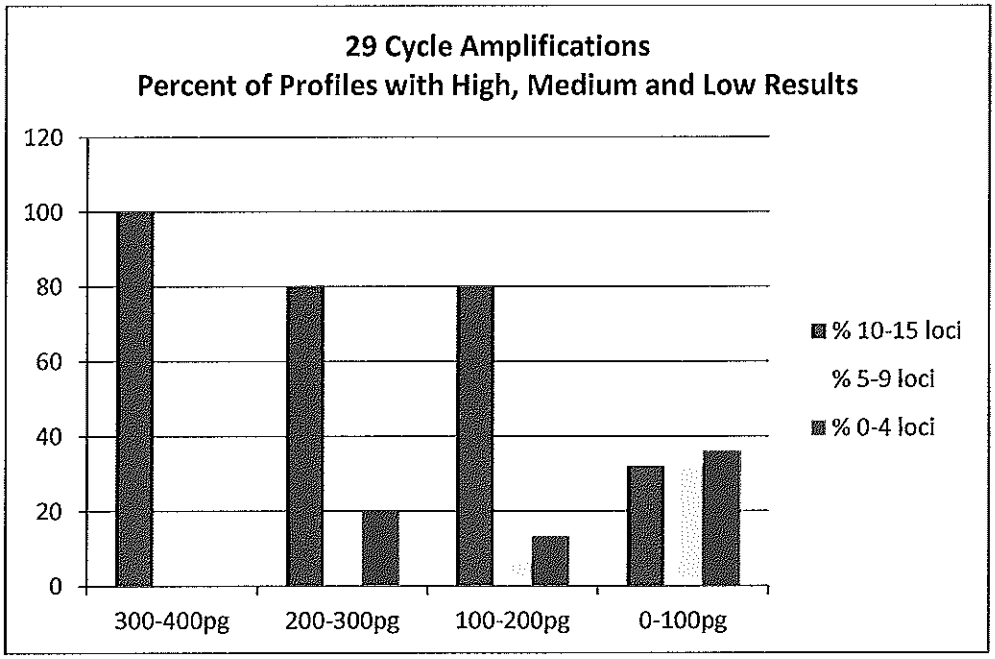

Figure 8. For samples amplified at greater than 100pg for 29 cycles, 80% or more of the profiles obtained were good (10-15 loci). For samples amplified at less than 100pg for 29 cycles, one-third of the profiles obtained were poor.

e. Conclusions

i. 28 Cycle Amplifications

Full profiles and profiles with no results were obtained from all quantities amplified below 400pg. At 200pg an overall shift from majority good profiles to majority poor profiles was observed. Average peak heights fall below stochastic threshold (200rfu) at less than 300pg. This is consistent with the Identifiler® Plus validation in which the minimum peak height of samples amplified with 250pg was 174rfu. High standard deviations in the number of loci obtained for each sample indicates that amplified quantities of 100-400pg may produce full profiles as low as 24% of the time. With two standard deviations, it can be expected for samples amplified with 50-100pg to produce profiles with anything from no results to 12 loci (with two standard deviations). Samples amplified with 0-50pg may produce profiles with no results or up to seven loci. Dropout was observed in samples amplified at 400pg or less. This is not consistent with the Identifiler® Plus validation in which dropout was not observed until the 125pg target, however may be attributable to the known differences between casework samples of unknown quality and high quality validation samples.

While the results of this study appear to produce somewhat consistent and expected averages, there are more than a few samples in each category of

amplification target which did not produce expected results. Many were shown to contain inhibition after amplification which was not indicated in the quantification procedure due to either the type or amount of inhibitor. Therefore, the actual concentrations of these samples as reported may not be accurate. Of the 18 samples which produced good and useable profiles, but quantified below 50pg, only six showed indications of inhibition.

An additional note should be included regarding the use of the Quantifiler Duo kit. It is not only important for a quantification kit to be consistent between lot numbers, but also between laboratory analysts. With Quantifiler Duo, as much as a two-fold difference can be expected in concentration values depending on analyst technique, kit lot, standard preparation date, etc. In addition, the value at which the quantification kit would be required to estimate is well below the sensitivity level established by internal validation. This may account for some of the high standard deviations obtained in this evaluation and the range of expected results from the different quantities. Therefore, it is recommended to investigate and/or internally validate a more accurate, sensitive quantification system in order to establish a reliable cutoff range for all samples prior to amplification.

i. 29 Cycle Amplifications

Samples amplified for an additional cycle produced more results at lower quantities than the 28 cycle amplifications. Improved profiles (greater number of loci with results) were observed for these samples. However, an increase in loci below stochastic threshold (300rfu) and a decrease in peak height ratios were also observed for these samples. This is consistent with the Identifiler® Plus validation and the expected effects of increasing cycle number.

Many of the samples evaluated for this study were previously amplified at 28 cycles and then amplified a second time with improved results at 29 cycles. Shortly after the 28 and 29 cycle validation of the Identifiler® Plus amplification kit, all 29 cycle amplifications were suspended. Therefore, a limited amount of 29 cycle samples were able to be assessed for this study. Additional data is needed to establish an appropriate amplification cut-off for a 29 cycle parameter.

II. Amplification Sensitivity

a. Objective

Determine a level of quantification at which useful results cannot be obtained from amplification.

b. Materials and Methods

Six samples were quantified with Plexor® HY to determine an approximate concentration. Four samples were diluted to approximate concentrations of 0.0325ng/µl, 0.03 ng/µl, 0.0275 ng/µl, 0.025 ng/µl, 0.0225 ng/µl, 0.02 ng/µl, 0.0175 ng/µl, 0.015 ng/µl, 0.0125 ng/µl, 0.01 ng/µl, 0.0075 ng/µl, 0.005 ng/µl, and 0.0025 ng/µl. Each dilution was quantified in triplicate with Plexor® HY and 10µl amplified in triplicate with Identifiler® Plus.

An additional two samples were diluted to the following approximate concentrations: 0.0150ng/µl, 0.0125 ng/µl, 0.0100 ng/µl, 0.0075 ng/µl, 0.0050 ng/µl, 0.0025 ng/µl, 0.0020 ng/µl, 0.0015 ng/µl, 0.0010 ng/µl, 0.0005 ng/µl, 0.00025 ng/µl, 0.000125 ng/µl, and 0.0000625 ng/µl. Each dilution was quantified in triplicate with Plexor® HY and 10µl amplified in triplicate with Identifiler® Plus .

All samples were run on a 3130xl and analyzed using GeneMapper® ID-X v1.3 with a 70rfu analytical threshold. See Appendix B for setup and results worksheets and sample electropherograms.

c. Data Analysis

Quantitation results were exported and an average value calculated for each set of triplicate samples using a Microsoft Excel spreadsheet.

Electropherograms were printed and assessed visually. The number of loci with a single allele below 200rfu (excluding Amelogenin) were counted and recorded for each sample. These numbers were then averaged by Microsoft Excel for each set of triplicate samples.

Sample results were exported from GeneMapper® ID-X to a Microsoft Excel spreadsheet as a combined table. The number of loci with results (excluding Amelogenin) was counted and averaged by Microsoft Excel. Average peak heights for each sample were also calculated by Microsoft Excel with all homozygous alleles divided in half. The average peak heights and standard deviations were then calculated for each set of triplicates.

Each set of samples was sorted by sample name (dilution number) and results evaluated.

See sample set-up worksheets and electropherograms for information regarding samples not used for analysis and/or re-injections.

d. Results

All controls (positives and ladders) produced appropriate genotypes and negative controls (negatives and NTCs) produced no results. Concordance for samples was checked and accuracy was confirmed by visual examination in Microsoft Excel. No discordant genotypes were observed.

District of Columbia
Department of Forensic Sciences
Forensic Science Laboratory
Forensic Biology Unit

Amplification Cutoff Validation

Sample Name	Amp Target (pg)	Average Number of Loci	Number of loci with one allele <200rfu	Average Peak Height
1-6A	366.649874	15	0	272.75
1-6B	324.043682	15	0	264.34375
1-6C	415.148407	15	0	276.2708333
1-6D	319.847866	15	0	214.875
1-6E	255.250342	15	0	206.1354167
1-6F	219.520984	15	0	214.46875
1-6G	220.138919	15	1.333333333	151.6770833
1-6H	187.742743	15	1	148.171875
1-6I	157.132701	13.66666667	5.333333333	124.4513889
1-6J	142.376788	12.66666667	3.333333333	121.9761905
1-6K	121.371013	11	6.666666667	102.3873626
1-6L	70.0865319	5.666666667	3.666666667	99.44907407
1-6M	49.8135035	1.333333333	1.333333333	88.66666667
2-10A	58.313688	15	2	147.4479167
2-10B	53.7794914	15	3.333333333	120.9270833
2-10C	38.8022817	13.33333333	7.666666667	105.0833333
2-10D	24.4795604	8.333333333	6	83.95416667
2-10E	24.1961331	5.333333333	5.333333333	68.11616162
2-10F	11.513232	2.666666667	2.666666667	79.41666667
2-10G	7.42618437	0	0	0
2-10H	5.12462142	0.333333333	0.333333333	37.5
2-10I	5.84062537	0	0	0
2-10J	2.21459088	0	0	0
2-10K	0.72726867	0	0	0
2-10L	0	0	0	0
2-10M	0.38666316	0	0	0
5-37A	313.139493	14.66666667	2.333333333	134.0952381
5-37B	273.045008	14.33333333	2.333333333	140.7916667
5-37C	277.988565	15	3.333333333	126.0520833
5-37D	242.64769	13.66666667	5.666666667	112.8456731
5-37E	210.176502	12	5.333333333	106.7364268
5-37F	207.009343	10	5	104.463955
5-37G	165.581124	10	7	95.89393939
5-37H	141.482024	5.666666667	3.666666667	91.91944444
5-37I	134.901204	6	4	87.85714286
5-37J	126.578109	5.666666667	2.666666667	82.31111111
5-37K	118.324563	5.666666667	3.333333333	100.5416667
5-37L	85.4559293	0.666666667	0.666666667	92.5
5-37M	59.3675662	0	0	0

Sample Name	Amp Target (pg)	Average Number of Loci	Number of loci with one allele <200rfu	Average Peak Height
BUCCALD-5A	283.201399	15	0	258.875
BUCCALD-5B	252.223189	15	0	301.96875
BUCCALD-5C	259.255094	15	0	223.0208333
BUCCALD-5D	173.237287	15	0.333333333	167.6875
BUCCALD-5E	168.972526	15	0	187.4479167
BUCCALD-5F	169.835912	15	1.333333333	156.6875
BUCCALD-5G	118.382053	15	2	147.5104167
BUCCALD-5H	109.587292	15	4	112.5666667
BUCCALD-5I	97.0770431	13	5.5	104.8461538
BUCCALD-5J	81.2762574	10	7	96.10378788
BUCCALD-5K	89.3643767	9.5	7	101.1666667
BUCCALD-5L	37.2990851	2.333333333	2.333333333	65.77777778
BUCCALD-5M	29.1873399	0	1	53.16666667
Q11B	78.7751005	15	1	105.4996947
Q11C	71.8390004	11.66666667	4.333333333	166.2604167
Q11A*	55.4697961	10.66666667	8	122.1902778
Q11D	45.8691745	11.66666667	7	106.5617716
Q11E	30.0394478	9.666666667	7.666666667	89.49768519
Q11F	14.8410065	1	0.666666667	82.625
Q11G	14.685824	3.333333333	1.333333333	81.16666667
Q11H	15.5698205	0	0	0
Q11I	8.72084917	0	0	0
Q11J	2.39408808	0	0	0
Q11K	2.38035659	0	0	0
Q11L	0.88338406	0	0	0
Q11M	0	0	0	0
Q15A	248.170005	15	0	295.34375
Q15B	231.210365	15	0	264.0416667
Q15C	196.099678	15	0.333333333	218.03125
Q15D	110.212329	15	0	234.3333333
Q15E	132.146985	15	0.333333333	191.25
Q15F	120.622595	15	0.666666667	187.375
Q15G	125.728726	15	2.333333333	138.96875
Q15H	141.228152	13.66666667	3.333333333	136.0194444
Q15I	83.785145	11.66666667	6.666666667	101.2261905
Q15J	43.2618088	11.33333333	7	114.3854167
Q15K	60.394929	10.33333333	7.666666667	85.40873016
Q15L	44.2472521	8	7	74.72857143
Q15M	14.5730492	0.666666667	0.666666667	83.5

* = dilution A prepared incorrectly and actually belongs between dilution C and D.

55.57550212 pg Average Amp Target Cut-off

10-15 loci
5-9 loci
0-4 loci
>50% of loci below stochastic threshold
amp quantity at which results change from high to medium/poor

Figure 8. Compiled results from all samples sorted by sample name. Each sample began to show signs of dropout at different levels. The overall average of the amplification targets at which high results profiles became medium/poor results profiles was 56pg.

Amplification Target	% 10-15 loci	% 5-9 loci	% 0-4 loci
>400	100	0	0
350-400	100	0	0
300-350	100	0	0
250-300	100	0	0
200-250	100	0	0
150-200	100	0	0
100-150	69.2307692	15.38462	15.38462
50-100	76.9230769	7.692308	15.38462
0-50	14.8148148	11.11111	74.07407

Figure 9.

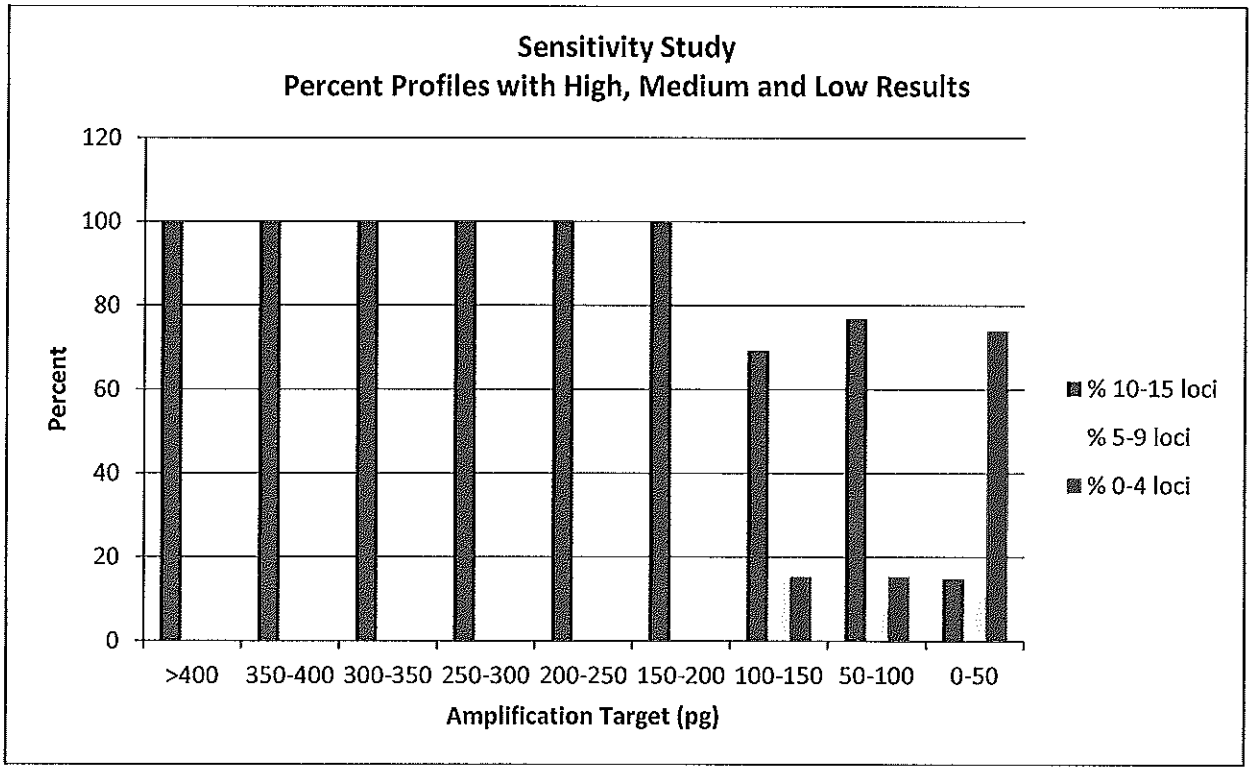

Figure 10. The majority of samples produced good profiles if amplified at more than 50pg. At 50pg or less, the majority of samples produced poor profiles. This is a lower quantity than was observed in the Casework Study.

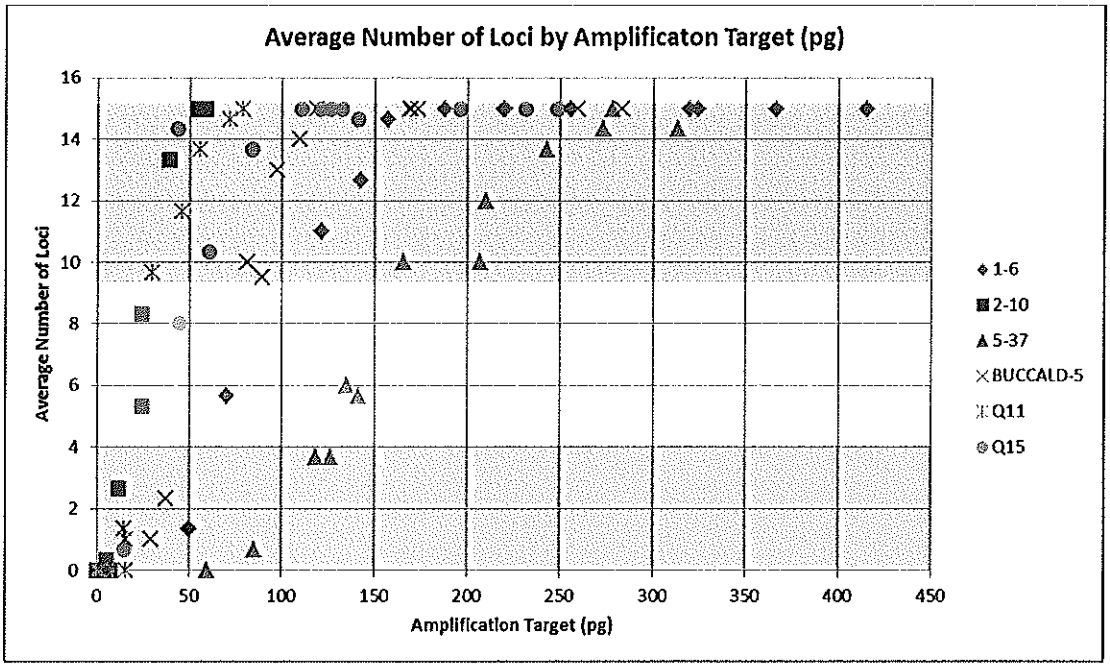

Figure 11. The red, yellow and green areas on the graph correspond to good, useable, and poor results profiles. Sample 5-37 produced fewer results for each amplification target than any of the other samples. All other samples produced good results until the 50-100pg range.

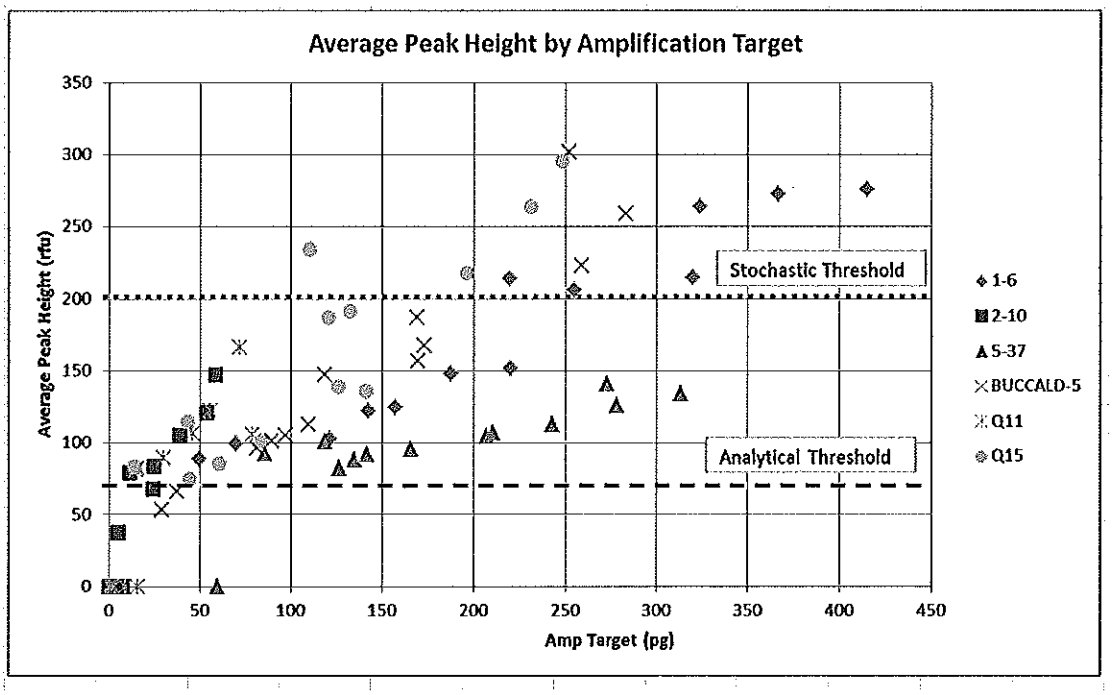

Figure 12. The majority of samples amplified under 300pg produced peak heights in the range between the laboratory's currently validated stochastic and analytical thresholds. Sample 5-37 produced lower overall peak heights than all other samples.

e. Conclusions

The previous study recommended a change in quantitation kit in order to appropriately establish a reliable amplification cutoff. Please refer to the Plexor® HY validation for verification of a quantitation kit capable of detecting DNA extract concentration down to a level of 0.865pg/µl with reproducible values obtained to a level of 2pg/µl. These values are less than the suggested amplification cutoff below.

In comparison to the casework portion of this validation, approximately 50pg was obtained for the shift from good to medium results profiles. If sample 5-37 is not included in this calculation, a value of approximately 40pg is obtained. This is a much lower quantity when compared to the casework quantity (using Quantifiler Duo), however it is consistent with the expected effect of switching quantitation kits to Plexor HY demonstrated in the Accuracy Study of the validation. In this study, the NIST standard samples and non-probative samples generally quantified lower using the Plexor® HY kit than the Quantifiler® Duo kit. It is also expected that single source samples would show better results at lower template amounts than the casework study samples which included single source and mixture profiles.

In order to determine an appropriate minimum quantity for samples to be amplified, it is important to include the expected variability (three standard deviations) of quantitation values at the calculated average amplification cutoff, 55.58pg. This value will be based on results from the Reproducibility/Sensitivity Study of the Plexor® HY Validation. The standard deviation is calculated from the average quantities and standard deviations obtained from two sets of dilutions quantified in triplicate on one plate and again by another analyst on a second plate. Using a best fit line, the average amplification cutoff value obtained from this validation, 55.58pg, can be expected to have a standard deviation of 4.859. Three standard deviations less, results in a 41pg minimum for amplification. This will be the recommended minimum value for samples to be amplified.

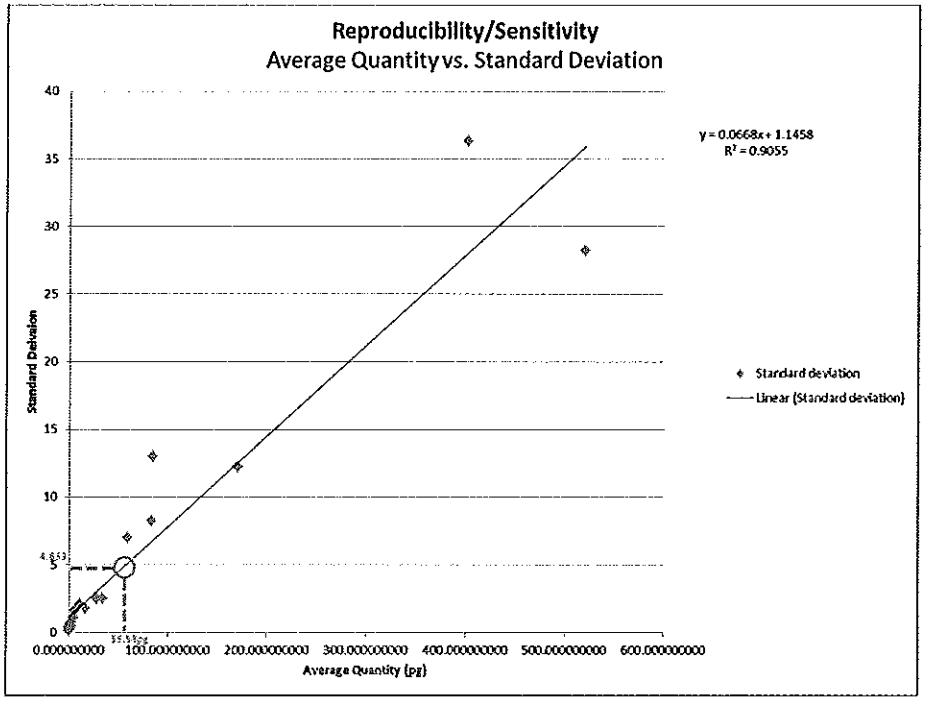

Figure 13.

In comparison to the Identifiler® Plus validation, this study produced similar values for peak heights and allelic dropout. The original validation showed initial dropout occurring at approximately 125pg and the samples of this study showed dropout at the following amplification targets: 157pg, 39pg, 313pg, 110pg, 72pg, and 141pg. The average of these values is 139pg. If sample 5-37 is not included in this calculation, the average is 104pg.

It is important to note that while samples amplified above the amplification cutoff may be expected to produce some dropout, low peak heights and peak height ratios, the laboratory has validated new probabilistic genotyping software, STRmix™. This software is able to model for the possibility of dropout and increased peak height variability for low level samples within its deconvolutions and statistical calculations.

III. Mixture

a. Objective

Determine a ratio of Total DNA to Male DNA at which no useable minor profile is detected.

b. Materials and Methods

Four male and four female samples were quantified with Plexor® HY to determine an approximate concentration. Samples were chosen in order to maximize the number of loci without shared alleles. Mixtures were made at total to male DNA ratios of 7:1, 10:1, 15:1, 20:1, and 25:1 with target concentrations of 0.5ng/µl, 1ng/µl, 5ng/µl, 10ng/µl and 15ng/µl. Each sample was quantified in triplicate with Plexor® HY. Mixture A was prepared and amplified in triplicate with a 1ng target in Identifiler® Plus. Peak heights were determined to be lower than expected for this mixture. Mixtures B, C and D were amplified at a 2ng target in Identifiler® Plus.

All samples were run on a 3130xl and analyzed using GeneMapper® ID-X v1.4 with a 70rfu analytical threshold. See Appendix D for setup and results worksheets and sample electropherograms.

c. Data Analysis

In order to prepare the mixtures, each sample (Male A, Male B, Male C, Male D, Female A, Female B, Female C, Female D) was quantified and then mixtures were calculated using a Microsoft Excel spreadsheet.

Each mixture was then prepared and quantified in triplicate using Plexor HY. Quantification results were evaluated in the Plexor Analysis Software to confirm standard curve and control values. Mixture sample results were then exported and an average value was calculated for each set of triplicate samples using a Microsoft Excel spreadsheet. The data was then sorted and an average value was calculated for each prepared ratio (7:1, 10:1, 15:1, 20:1, 25:1).

Each mixture was also amplified in triplicate using Identifiler Plus and run on the 3130xl Genetic Analyzer. Results were analyzed in GeneMapper® ID-X Version 1.4 and evaluated. Appropriate profiles for controls were verified and artifacts (spikes, background, pull up, etc.) were identified and removed.

Mixture sample results were then exported from GeneMapper® ID-X to a Microsoft Excel spreadsheet as a combined table. The number of detected alleles (excluding

Amelogenin) was counted and subtracted from the expected number of alleles to determine dropout. Average dropout was then calculated for each mixture ratio. The average ratio of total DNA to male DNA for each mixture was also calculated using the peak heights of loci where no alleles were shared. The sample ratios were then averaged to determine an overall total DNA to male DNA ratio for each mixture.

See sample set-up worksheets and electropherograms for information regarding samples not used for analysis and/or re-injections.

d. Results

i. Quantitation

		A		B		C		D	
Expected Ratio	Quantity (ng)	Mix A Average Ratio	Standard Deviation	Mix B Average Ratio	Standard Deviation	Mix C Average Ratio	Standard Deviation	Mix D Average Ratio	Standard Deviation
	0.5	14.96896	3.68815	16.0605	0.816139	10.88155	0.310886	11.60872	1.107261
	1	23.18088	0.922037	24.46753	9.402203	8.766756	0.814378	12.1162	0.526778
	5	18.83539	1.401104	12.62823	0.869679	8.057176	0.218555	10.78982	1.915571
	10	22.18867	2.61669	12.59378	1.263306	6.951642	0.072299	8.930957	0.962918
7	15	22.32287	1.719082	14.76271	2.453497	6.676053	0.495368	10.53033	0.137283
	0.5	23.95102	1.226619	23.58558	1.492098	17.18752	2.670017	17.74401	0.351918
	1	31.70781	1.198737	19.52476	4.961417	15.70213	1.977166	18.55011	1.883349
	5	31.09398	1.293373	21.02884	0.88041	14.06673	1.100361	19.08914	1.044491
	10	31.01452	2.410064	19.69336	1.505415	12.40175	0.866895	11.97475	0.490799
10	15	38.27938	1.115146	20.46776	2.663712	10.11372	0.64927	16.04292	2.827302
	0.5	40.96884	6.536695	37.83552	7.104782	22.23731	2.977805	25.83018	6.672995
	1	53.87513	7.375943	34.48286	10.62953	23.97139	3.956519	27.20018	5.042903
	5	56.28943	5.996079	31.90343	1.68775	24.6976	1.64568	33.25591	4.123558
	10	59.95287	3.966677	33.0867	1.657389	24.34259	4.516805	24.62166	3.894013
15	15	62.63766	13.6609	35.64256	8.485626	21.61498	3.044649	28.72323	2.710584
	0.5	52.51915	14.72206	40.13367	6.060236	38.48264	10.26107	35.81665	4.717465
	1	66.22263	8.840219	53.75912	13.29867	31.75989	5.478223	41.94905	5.109899
	5	69.85034	12.46631	51.2805	1.76985	33.28636	1.72853	37.2845	1.464143
	10	77.92514	8.240042	57.28258	7.546687	34.08308	1.191325	36.37494	4.534351
20	15	93.72321	25.94596	50.58193	5.446491	39.73839	6.023768	38.81672	1.316815
	0.5	60.77257	9.1372	58.47198	7.79773	45.18467	4.719161	43.72024	8.20466
	1	79.82771	17.38111	65.35989	5.685596	41.3839	2.487526	50.0142	5.627141
	5	92.05865	6.105572	60.6588	3.804194	39.58662	0.90633	54.15574	4.168319
	10	92.06361	14.50873	70.43411	14.40373	43.8675	6.947279	44.24204	6.745869
25	15	107.0107	32.20182	76.0654	22.98109			42.26951	2.889269

Figure 14. This table shows the average ratio determined by quantitation for each target ratio at the 5 different input quantities. An overall trend of higher than expected ratios was observed for all mixtures at all quantities.

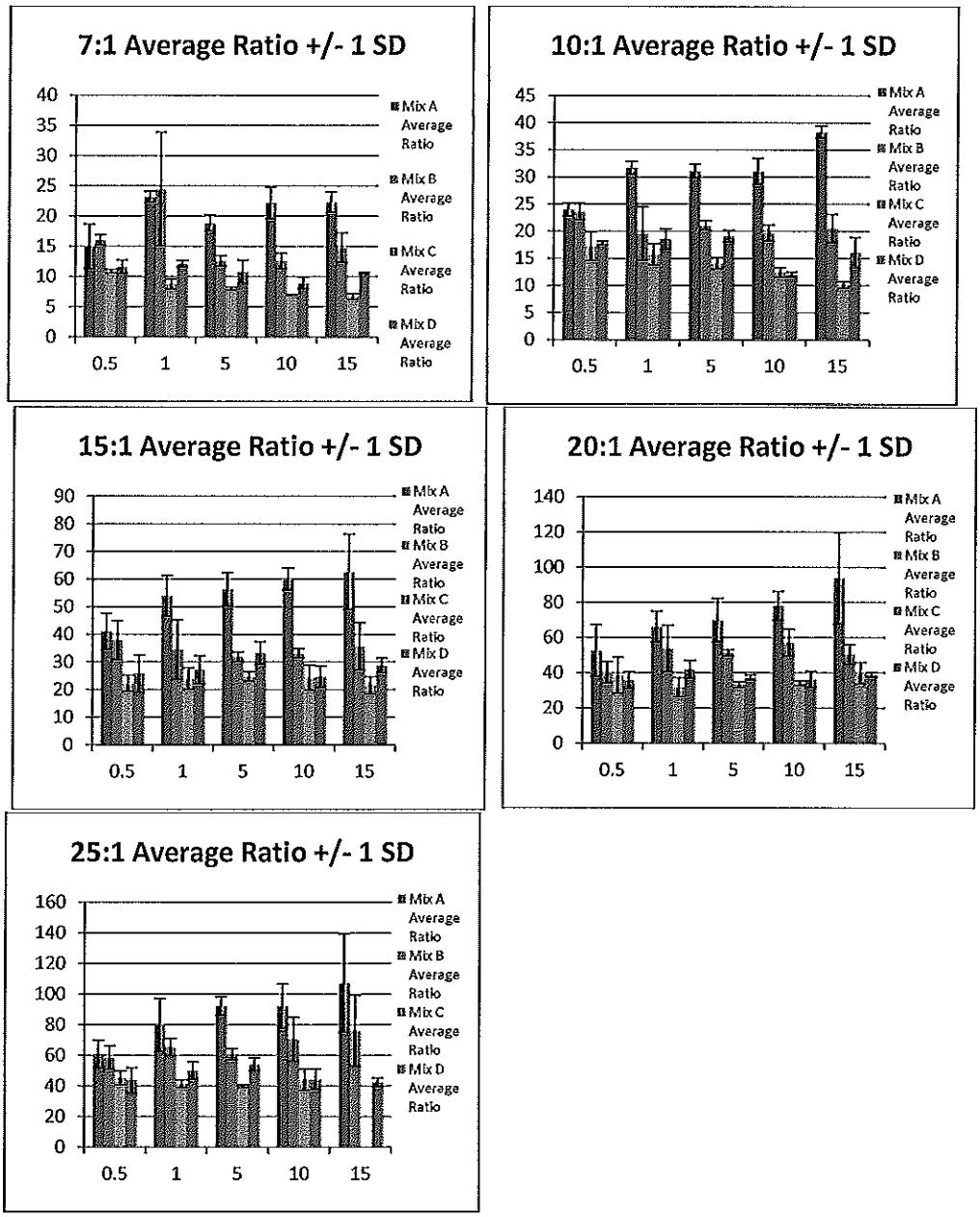

Figure 15. For Mixture A, the average ratio increased with an increase in the amount of DNA added to the quantitation. However, no other mixtures displayed this same trend. Mixtures B, C and D produced consistent ratios despite quantity.

	Mixture A		Mixture B		Mixture C		Mixture D		Overall
Expected Ratio	Average Observed Ratio	Standard Deviation	Average Observed Ratio	Standard Deviation	Average Observed Ratio	Standard Deviation	Average Observed Ratio	Standard Deviation	Average Observed Ratio
7:1	20.29935406	3.7033466	16.10254917	5.875708062	8.266635213	1.609996855	10.79520717	1.466175121	13.8659364
10:1	31.20934151	4.871474417	20.86005985	2.754511319	13.89436926	2.913456466	16.68018528	2.987215415	20.66098898
15:1	54.74478577	10.41257113	34.59021142	6.242270815	23.3727739	3.117938224	27.9262313	5.025595519	35.15850059
20:1	72.04809545	19.1912652	50.6075591	8.866441241	35.4700718	5.947642544	38.04837472	3.947128533	49.04352527
25:1	86.34664187	22.21194603	66.19803563	12.8328834	42.5056723	4.385111568	46.88034637	6.77863529	60.48267404

Figure 16. This table calculates an overall observed ratio for each mixture. This calculation is appropriate since the previous data demonstrated no affect by the total input quantity for Mixtures B, C and D.

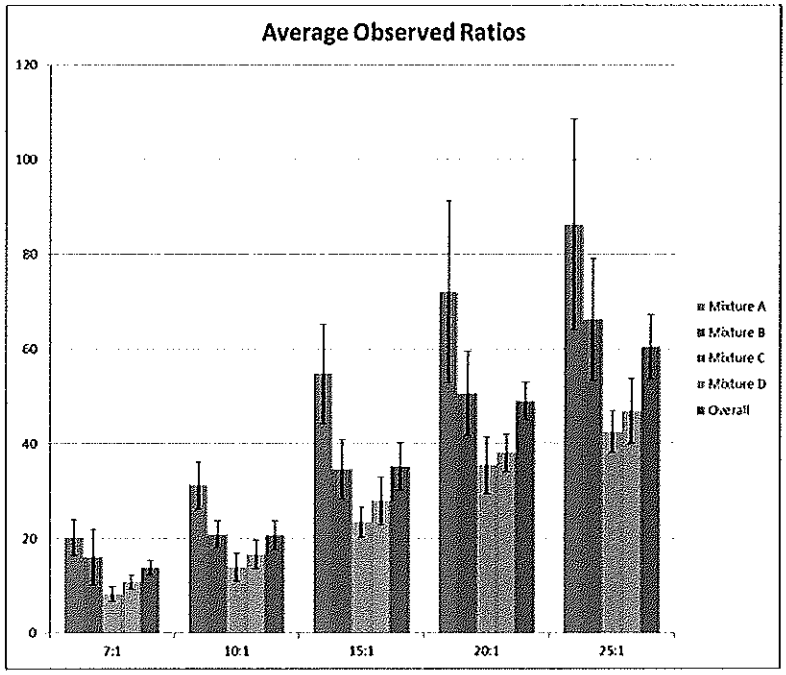

Figure 17. This figure shows an overall trend of higher than expected ratios. In order to determine whether this trend is due to the quantification process or a poor initial quant value obtained prior to dilution, all samples were amplified and mixture ratios calculated.

ii. Amplification

Average Number of Missing Alleles

Expected Ratio	Mixture A (1ng)	Mixture B (2ng)	Mixture C (2ng)	Mixture D (2ng)	Average (excludes Mixture A)
7:1	6.2	0.133333333	0	0.466666667	0.2
10:1	12.53333333	2	0.4	1.533333333	1.311111111
15:1	17.06666667	5.466666667	4	4.333333333	4.6
20:1	20.13333333	10.2	6.076923077	7.066666667	7.781196581
25:1	21.46666667	12.53333333	9.25	11.26666667	11.01666667

Figure 18. This table shows the average amount of dropout for Mixtures B, C and D. Due to a lower than ideal target input of 1ng, data from Mixture A will not be used for calculations in the amplification portion of the Mixture Study. Little to no dropout was observed in the 7:1 and 10:1 ratios. An average of five alleles was observed to dropout at the 15:1 ratio. Dropout in the 20:1 and 25:1 ratios indicates approximately half of the minor component missing (data does not include alleles which are shared by both contributors).

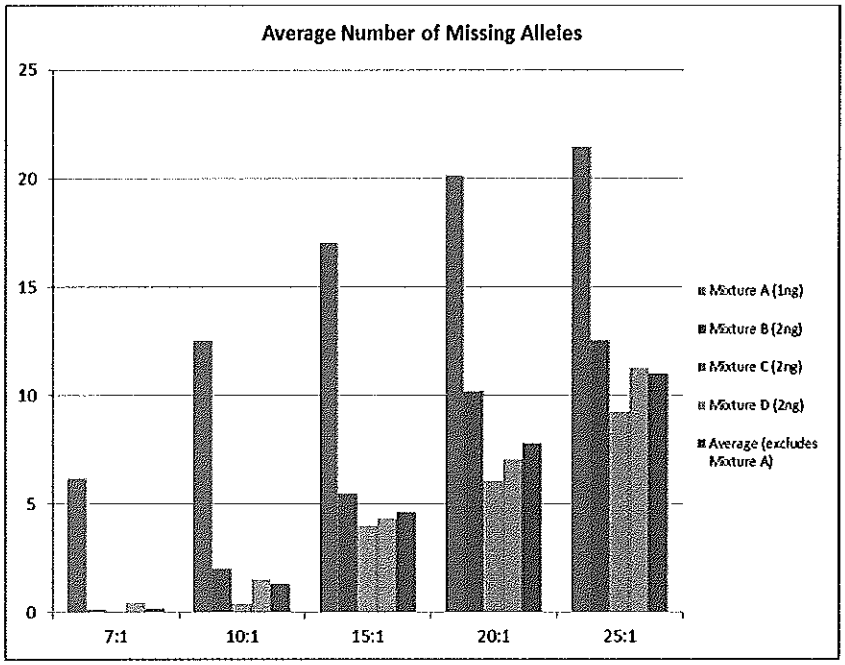

Figure 19. Similar values were obtained for Mixtures B, C and D. For Mixture A which was amplified at a lower input, a higher than expected number of missing alleles was obtained.

Average Male Heterozygote Peak Height

Expected Ratio	Mixture A (1ng)	Mixture B (2ng)	Mixture C (2ng)	Mixture D (2ng)	Overall Average (without Mixture A)
7	117.8693182	180.5298507	290.99	246.9179688	239.4792732
10	95.03191489	134.9188034	224.0472973	169.8628319	176.2763109
15	75.20454545	103.2682927	173.6008772	131.8352941	136.2348213
20	75.45	71.04385965	150.8571429	97.64583333	106.5156119
25	58.1	58.29268293	128.3365385	69.46875	85.36599046

Figure 20. All mixtures produced average male peak heights above 70rfu down to a 20:1 mixture ratio.

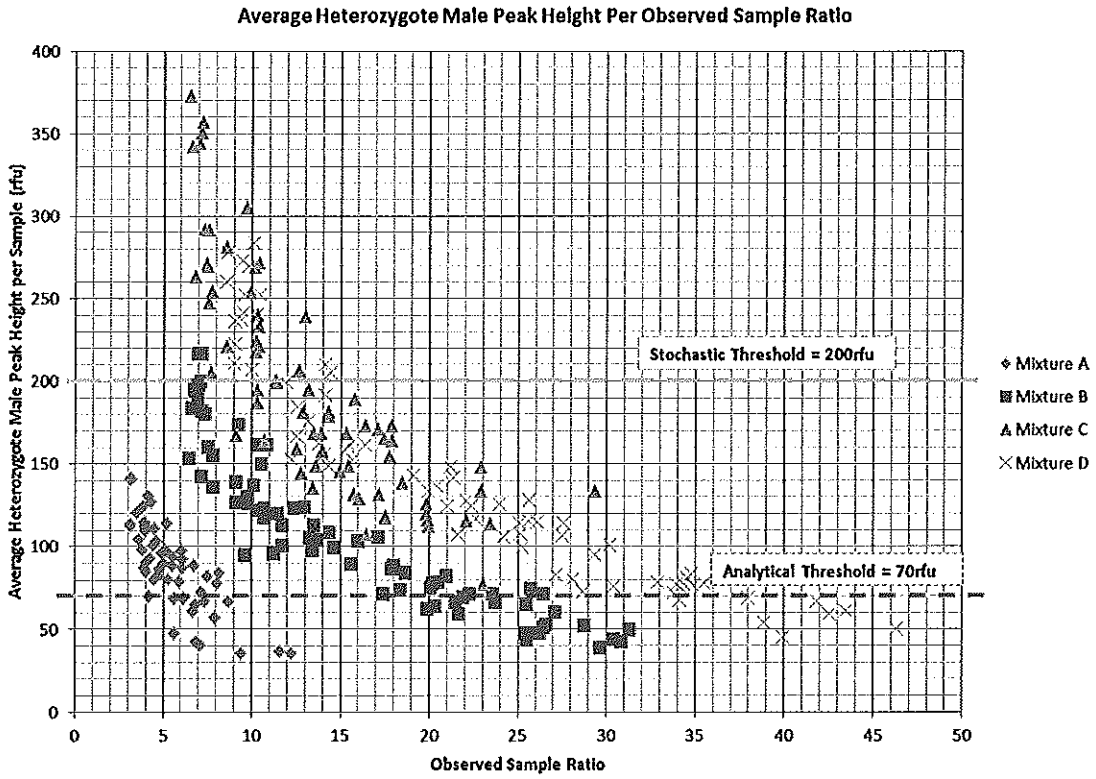

Figure 21. For this graph, average male peak heights for each sample were compared to the calculated overall ratio for each sample. Without considering Mixture A, the average male peak heights are above analytical threshold until the 20:1 ratio. The stochastic threshold line indicates that the majority of average peak heights are below stochastic threshold for all samples, however, some samples with ratios as low as 15:1 produced averages above 200rfu.

Average Total/Male per Ratio

Expected Ratio	Mixture A (1ng)	Mixture B (2ng)	Mixture C (2ng)	Mixture D (2ng)	Overall Average (without Mixture A)	Standard Deviation
7	3.936363596	7.103850459	7.424123349	9.5528848	8.026952869	1.331163
10	4.799832909	10.25822291	10.33254617	13.83801393	11.476261	2.0456756
15	6.793854932	14.49337759	13.73492323	21.95130527	16.72653536	4.5406474
20	6.452582384	21.47339957	17.21449342	29.43031169	22.70606823	6.2004966
25	8.871301291	26.47247711	21.01089224	36.44126951	27.97487962	7.8241323

Figure 22. Mixture A was not used for calculation of the average or standard deviation of the total/male per ratio. While Mixture D produced higher than expected ratios, all averages for Mixtures B, C and D fall within two standard deviations of the expected ratio. If a sample is amplified at a lower than ideal target (Mixture A), the calculated mixture ratio will be significantly lower than actually present.

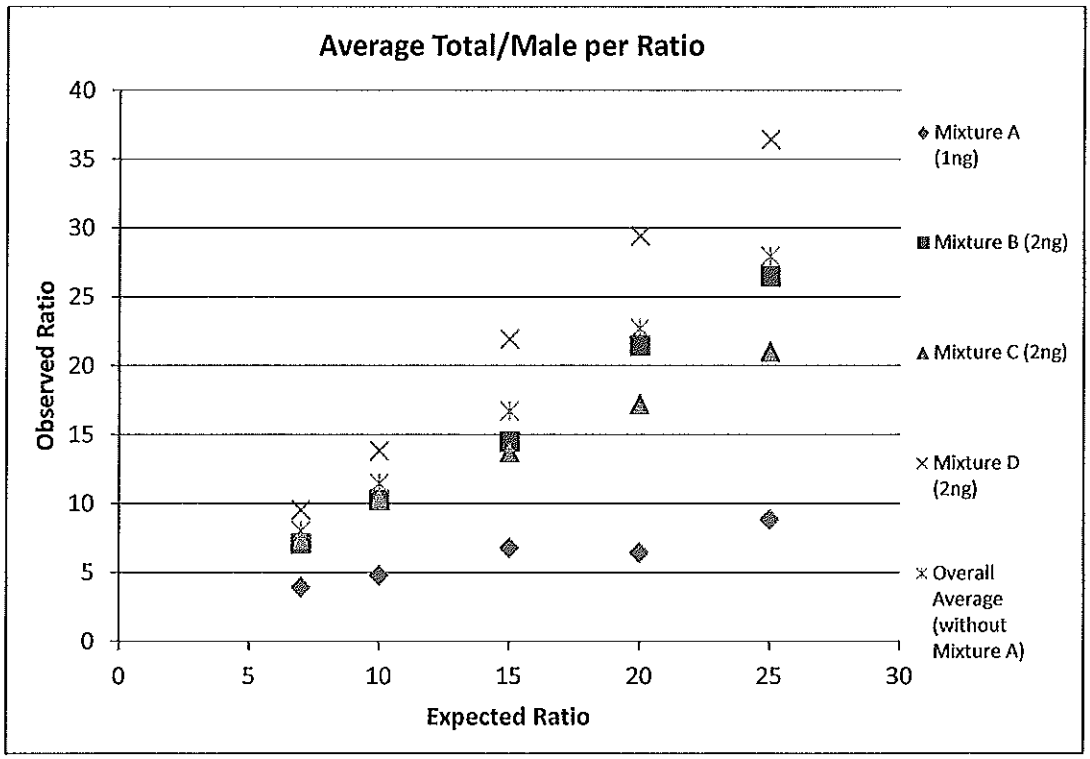

Figure 23. This graph demonstrates the stochastic effect of a mixture amplified at a lower than ideal target. Mixture A produced much lower ratios than expected, while Mixtures B, C and D produced mixtures which are similar to the expected. However, as the mixture ratio increases, more variability can be expected in the observed ratio.

e. Conclusions

Based on the results of the Mixture Study, significant dropout of the minor contributor can be expected to occur at amplification ratios of 25:1 or greater when a sufficient quantity of DNA is available for amplification. Due to the quantification process, the graph below shows an approximate two-fold difference between the estimated ratio of total to male DNA predicted by the quantification results and amplification results. Therefore, an appropriate amplification cutoff ratio for samples which are expected to have only two contributors with the majority from a female is a 45:1 ratio.

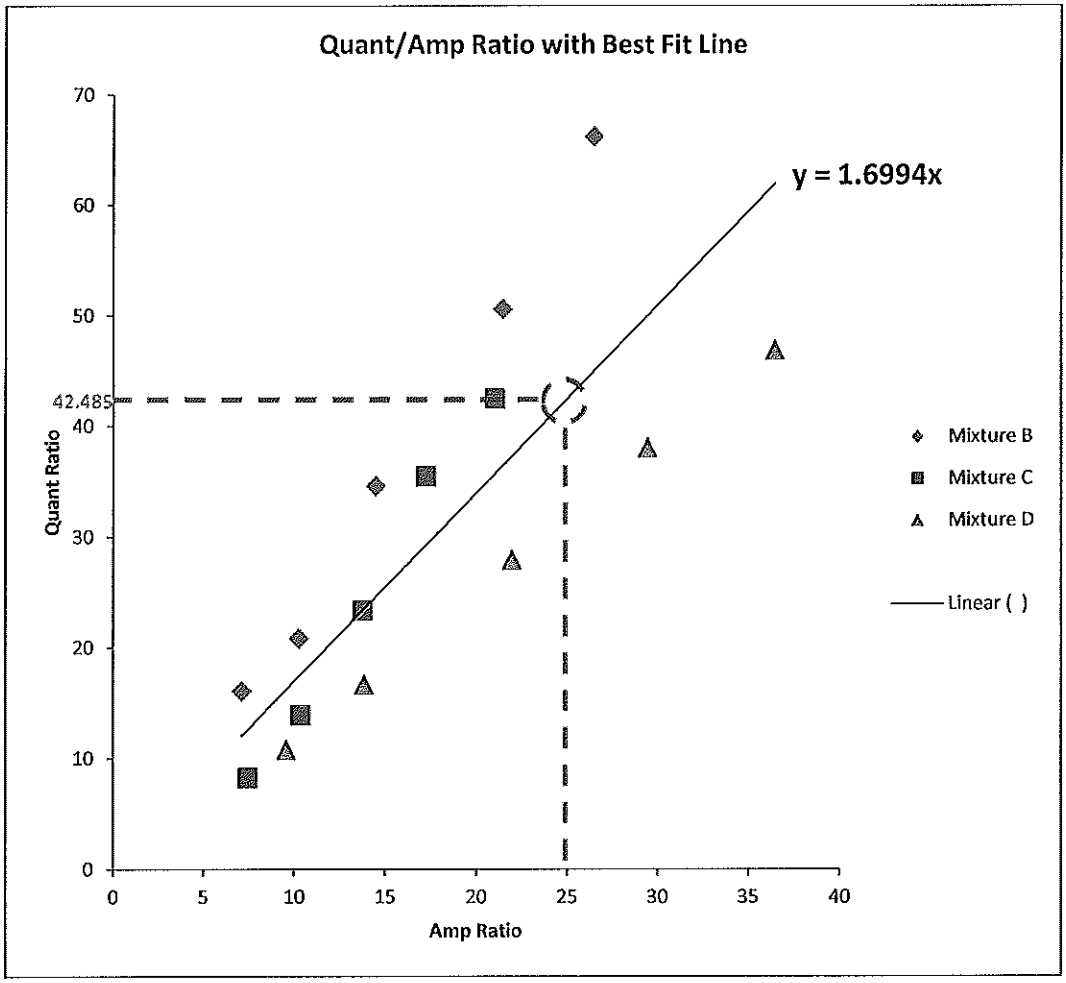

Figure 24.

While Mixtures B, C and D were used for all calculations, the results from Mixture A should still be considered when making decisions about samples which are to be amplified. If a mixture sample will not reach an appropriate target input, the expected number of missing alleles will be higher than expected. In addition, the mixture ratio calculated by peak heights may vary significantly from quantification results due to stochastic effects. Quantification results from two person mixture samples which fall below 0.2ng/µl and above the suggested amplification cutoff, 41pg, can be expected to produce significant dropout of the minor contributor at a higher ratio of total to male DNA than 45:1. Further sensitivity studies would be required to establish an appropriate cutoff for mixtures amplified at lower quantities.

Conclusion

Using the data obtained in this study, it is recommended to modify laboratory standard operating procedures to reflect a change in overall sample processing. If a sample quantifies at a value less than 4.1pg/µl using Plexor® HY as the quantitation kit, it may be stopped from further processing based on the assumption that the profile will exhibit significant dropout and/or loss of data.

For sexual assault samples with a sufficient quantity of total DNA to amplify an optimal target (≥500pg) where a probative, minor contributor male is expected, a quantification ratio of total DNA to male DNA greater than 45:1 is not expected to yield enough of a minor profile to be used for comparison. For mixture samples without a sufficient quantity to amplify an optimal target (<500pg), it is recommended to amplify as much quantity as possible regardless of the ratio indicated by Plexor HY unless other samples in the case have better potential for full results (higher amplification target and/or lower ratio).

Because the quantitation step will be used to stop samples from further processing, it is critical that as much consistency among analysts and kit lot numbers be maintained. If an analyst's standard curve does not fit within the validated range, it is possible the sample may quantify below the amplifiable range but be capable of producing full results. All samples should be re-quantified. Also, if a quantitation kit does not pass QC, a sensitivity study should be conducted and staff notified if a change in amplification cutoff is needed.